

Chemical Structure of the Lipid A Component of Lipopolysaccharides from *Fusobacterium nucleatum*

SUMIHIRO HASE, TOR HOFSTAD, AND ERNST T. RIETSCHEL*

Max-Planck-Institut für Immunbiologie, D-7800 Freiburg, Federal Republic of Germany; and Laboratory of Oral Microbiology, University of Bergen, Bergen, Norway*

Received for publication 20 July 1976

The lipid A component of lipopolysaccharides from *Fusobacterium nucleatum* Fev 1 consists of β -1',6-linked D-glucosamine disaccharides, which carry two phosphate groups: one in glycosidic and one in ester linkage. The amino groups of the glucosamine disaccharides are substituted by D-3-hydroxyhexadecanoic acid. The hydroxyl groups of the disaccharide backbone are acylated by tetradecanoic, hexadecanoic, and D-3-hydroxytetradecanoic acids. Part of the ester-bound D-3-hydroxytetradecanoic acid is 3-O-substituted by tetradecanoic acid. Whereas a similar pattern of fatty acids was detected in lipopolysaccharides from two other *F. nucleatum* strains, the amide-bound fatty acid in *F. varium* and *F. mortiferum* was D-3-hydroxytetradecanoic acid. The chemical relationships of lipid A from *Fusobacteria* and other gram-negative bacteria are discussed.

Lipopolysaccharides from *Fusobacterium* resemble those of aerobic gram-negative bacteria such as *Salmonella* in that they consist of a polysaccharide and a lipid component (14, 16). They appear in the electron microscope as structural particles (10) and possess O-antigenic specificity (15).

The endotoxic activity of *Fusobacterium* lipopolysaccharides appears to be comparable to that of *Salmonella* endotoxins (3, 4, 21). In *Salmonella* lipopolysaccharides (and those of other bacterial groups), the endotoxic activity is embedded in the lipid A component (3, 16, 21), the chemical structure of which has been studied (5, 6, 22).

The present communication deals with the chemistry of the lipid A component of *Fusobacterium nucleatum* lipopolysaccharides. It will be shown that its structure is related to that of *Salmonella* lipid A.

(Part of this work was presented at the Annual Meeting of the JADR, London [E. T. Rietschel, J. Minner, and T. Hofstad, J. Dent. Res., vol. 54, special issue A, abstr. L214], and at the 8th Meeting of the North West European Microbiological Group, Helsinki, 1976.)

MATERIALS AND METHODS

Bacteria. *F. nucleatum* strain Fev 1 (1) was kindly provided by S. E. Mergenhagen (Bethesda, Md.). The origin of the other strains (*F. nucleatum* F1 and ATCC 10953, *F. mortiferum* VIP 0473, and *F. varium* ATCC 8501) has been described previously (8, 13).

Cultures were grown in screw-cap bottles filled to the top with the following (in grams per liter): tryptone (Oxoid), 15; proteose peptone (Oxoid), 10; NaCl, 5; KH_2PO_4 , 1.5; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3.5; $(\text{NH}_4)_2\text{SO}_4$, 0.5; yeast extract (Oxoid), 3; L-cysteine · HCl, 1; and glucose, 2.5; pH 7.0. Cultures were grown for 48 h and washed two times with phosphate-buffered saline (pH 7.4).

Lipopolysaccharides. Washed organisms were extracted with phenol-water (25) at 20°C, and the lipopolysaccharide was purified by ultracentrifugation ($100,000 \times g$, 90 min) and treatment with deoxyribonuclease and ribonuclease (9).

Reference compounds, chemicals, and enzyme. Tetradecanoic, hexadecanoic, and heptadecanoic acids were obtained from Serva (Heidelberg). 3-Hydroxytetradecanoic and 3-hydroxyhexadecanoic acids were synthesized by the Reformatzky reaction (22). 3,4,6-Tri-O-methyl-1,5-di-O-acetyl-2-deoxy-2-(N-methylacetamido)-glucitol was prepared from methyl-N-acetylglucosaminide by permethylation, followed by acid hydrolysis, reduction, and peracetylation (7). The optical antipodes (D- and L-forms) of the 3-hydroxy acids were prepared from the racemates with amphetamine according to Ikawa et al. (11). N-3-hydroxytetradecanoyl-D-glucosamine was obtained from G. van der Smissen (Freiburg), N-acetylglucosaminyl- α -1',4-N-acetylglucosamine from H. Paulsen (Hamburg), β -N-acetylglucosaminidase (EC 3.2.1.30; *Aspergillus oryzae* [18]) from Y. Matsushima and T. Mega (Osaka), and hydrazine from Roth (Karlsruhe).

Chemical analyses. (i) **Backbone.** The backbone of lipid A from *F. nucleatum* Fev 1 lipopolysaccharide was prepared as described for *Salmonella* and other bacterial groups (6). Briefly, lipopolysaccharide was first treated with alkali (0.17 N NaOH, 60

min, 100°C) and then with acid (0.02 N HCl, 35 min, 100°C). The precipitate formed, representing alkali-treated lipid A (lipid A-OH), was reduced with NaBH₄ (lipid A-OH_{red}) and treated with hydrazine (40 h, 100°C). The reduced lipid A backbone consisting of a reduced glucosamine disaccharide (GlcN-GlcN_{red}) was isolated by high-voltage electrophoresis and *N*-acetylated (GlcNAc-GlcNAc_{red}).

Methylation analysis of the reduced and *N*-acetylated backbone by gas-liquid chromatography as well as enzymatic analysis with β -*N*-acetylglucosaminidase was carried out as described previously (6, 7).

(ii) **Fatty acids.** Ester-bound fatty acids were released from lipopolysaccharides with sodium methylate (0.25 N NaOCH₃, 10 h, 37°C), and amide-bound fatty acids were released from the de-*O*-acylated preparation by strong alkaline hydrolysis (4 N KOH, 5 h, 100°C). For estimation of total fatty acids, lipopolysaccharide was treated with 4 N KOH (5 h, 100°C). The acids were analyzed by gas-liquid chromatography in the form of the methyl esters (22). Two types of columns (glass) were used: Castorwax (2.5% on Chromasorb G, 80 to 100 mesh) at 175°C and an ethylene succinate-methylsilicone copolymer (EGSS-X, 15% on Gas-Chrom P, 100 to 200 mesh) at 140°C. The nature of fatty acids was determined by comparison of their retention times (*t_R*) obtained on both columns with those of authentic reference fatty acids and by their fragmentation pattern obtained on combined gas-liquid chromatography-mass spectrometry. *n*-Heptadecanoic acid methyl ester served as an internal standard for quantitative assays.

Configuration analysis of 3-hydroxy acids was performed by gas-liquid chromatography (3% OV-1 on Gas-Chrom Q, 100 to 200 mesh, 205°C) of their diastereomeric 3-methoxy-*L*-phenylethylamides (20).

Other methods of analysis. Colorimetric determinations of total phosphate and hexosamine (with and without hydrolysis), and other analyses including high-voltage paper electrophoresis, gas-liquid chromatography (Varian 1400), mass spectrometry (Finnigan 3200), and assays with an amino acid analyzer (Durrum, model D-500), were carried out as described previously (6, 7).

RESULTS

Backbone. To elucidate the structure of the lipid A backbone, essentially the procedure described previously was followed (6). *F. nucleatum* Fev 1 lipopolysaccharide (46 mg) was treated with alkali (saponification of ester-bound fatty acids) and acid (hydrolysis of polysaccharide and glycosidically linked phosphate). The precipitate formed (lipid A-OH, 20% yield) contained glucosamine and phosphate in a molar ratio of 2.1:1.0. In the direct Morgan-Elson reaction (without hydrolysis), using *N*-3-hydroxytetradecanoyl glucosamine as a standard, the color yield corresponded to 0.74 mol of glucosamine (per 2 mol of glucosamine), indicating that part of the glucosamine

in lipid A-OH is reducing. Lipid A-OH was then reduced (NaBH₄), yielding lipid A-OH_{red} (73% yield). With the amino acid analyzer, glucosaminitol (0.45 μ mol/mg), glucosamine (0.17 μ mol/mg), and glucosamine-phosphate (0.24 μ mol/mg) could be detected. The molar ratio of glucosamine to phosphate was 1.03:1.0. As expected, lipid A-OH_{red} was negative in the direct Morgan-Elson assay.

Lipid A-OH_{red} was treated with hydrazine (40 h, 100°C) to remove amide-bound fatty acids and ester-linked phosphate groups, and the hydrazinolysate was subjected to high-voltage electrophoresis. With ninhydrin, one major fraction ($M_{GlcN} = 1.15$), corresponding to a glucosaminyl-glucosaminitol disaccharide (6, 7), and a second spot (approximately 10% of hydrazinolysate, $M_{GlcN} = 1.01$), corresponding to glucosaminitol, were detected. The disaccharide fraction was eluted with acid (0.01 N HCl) and *N*-acetylated (30% yield, relative to lipid A-OH_{red}, based on glucosamine).

Preparation GlcNAc-GlcNAc_{red} (0.94 μ mol) was methylated according to Stellner et al. (24). After hydrolysis, reduction, and peracetylation of the methylated material, gas-liquid chromatography gave three peaks (Table 1). Their relative *t_R* values indicated (7) that these peaks correspond to 1,3,4,5-tetra-*O*-methyl-6-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)-glucitol (peak 1, *t_R* = 0.43), 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)-glucitol (peak 2, *t_R* = 1.00), and 1,3,4,5-tetra-*O*-methyl-6-*O*-acetyl-2-deoxy-2-(*N*-acetylacetamido)-glucitol (peak 3, *t_R* = 1.10). The relative amounts of the peaks were 5% (peak 1), 53% (peak 2), and 41.8% (peak 3). It has previously been shown (7) that from 6-*O*-substituted glucosaminitol after methylation, hydrolysis, reduction, and peracetylation, both the *N*-meth-

TABLE 1. Identity, relative retention times, and relative amounts of derivatives formed on methylation analysis of reduced lipid A backbone (GlcNAc-GlcNAc_{red}) from *Fusobacterium nucleatum* Fev 1 lipopolysaccharide

Peak no. ^a	Identity	Relative <i>t_R</i> ^b	Relative amt (mol %)
1	1,3,4,5-Tetra- <i>O</i> -methyl-6- <i>O</i> -acetyl-2-deoxy-2-(<i>N</i> -methylacetamido)-glucitol	0.43	5.4
2	3,4,6-Tri- <i>O</i> -methyl-1,5-di- <i>O</i> -acetyl-2-deoxy-2-(<i>N</i> -methylacetamido)-glucitol	1.00	53.8
3	1,3,4,5-Tetra- <i>O</i> -methyl-6- <i>O</i> -acetyl-2-deoxy-2-(<i>N</i> -acetylacetamido)-glucitol	1.11	40.8

^a On gas-liquid chromatography.

^b Based on peak 2 (*t_R* = 1.00) on ECNSS-M.

ylacetamido- and the *N*-acetylacetamido derivatives are formed (in a ratio of about 1:10). Therefore, it was concluded that peaks 1 and 3 derive from the *N*-acetylglucosaminitol residue and peak 2 from the nonreducing *N*-acetylglucosamine residue of fraction GlcNAc-GlcNAc_{red}. The fact that the compounds corresponding to peaks 1 and 3 are 6-*O*-acetylated shows that in fraction GlcNAc-GlcNAc_{red} a 1',6-linkage is present.

To study the anomeric configuration of glucosamine, fraction GlcNAc-1',6-GlcNAc_{red} was treated with β -*N*-acetylglucosaminidase. The *N*-acetylglucosamine released, as estimated by the direct Morgan-Elson reaction, was 64% after 10 min and 94% after 60 min of incubation. These results agree with those of previous studies (6). Thus, the nonreducing α -glucosamine residue in fraction GlcNAc-1',6-GlcNAc_{red} is present as the β -anomer. Control experiments with chitobiitol and a reduced α -1',4-linked glucosamine disaccharide gave significantly lower rates and no release of glucosamine, respectively (6).

Fatty acids. Gas chromatographic analysis of fatty acids released from lipopolysaccharides of *F. nucleatum* Fev 1 by alkali (4 N KOH) revealed the presence of tetradecanoic, hexadecanoic, 3-hydroxytetradecanoic, and 3-hydroxyhexadecanoic acids in amounts of 0.258, 0.037, 0.204, and 0.110 μ mol per mg of lipopolysaccharide, respectively (Table 2). Also Δ^2 -tetradecenoic acid was detected. This acid, however, which was not present in acid hydrolysates, represents an artifact resulting from 3-hydrox-

ytetradecanoic acid (22). The total amount of fatty acids was 0.61 μ mol per mg of lipopolysaccharide (= 15 wt%). To distinguish between ester- and amide-bound fatty acids, lipopolysaccharide was first treated with sodium methylate. On gas-liquid chromatography of the methyl esters, the non-hydroxylated fatty acids (C_{14:0}, 0.03 μ mol/mg; C_{16:0}, 0.03 μ mol/mg) and 3-hydroxytetradecanoic acid (0.05 μ mol/mg) were found (Table 2). In addition, 3-methoxytetradecanoic acid methyl ester (0.079 μ mol/mg) was detected. Since this latter acid was not present in acid hydrolysates, it was concluded that it is an artifact resulting from a β -elimination reaction involving 3-*O*-substituted, ester-bound 3-hydroxytetradecanoic acid. The product of the β -elimination reaction is known to be Δ^2 -tetradecanoic acid methyl ester (as detectable in alkaline hydrolysates). Under the conditions of methylate treatment, however, a nucleophilic addition of methylate to the α,β -unsaturated acid ester takes place, yielding 3-methoxytetradecanoic acid methyl ester (22, 23).

The nature of the substituent at the 3-hydroxyl group of 3-hydroxytetradecanoic was established in a similar way as described for *Salmonella* (22); e.g., part of the methanolysate was carbomethylated with diazomethane and compared with the nontreated methanolysate (Table 2, A and B). The peak corresponding to tetradecanoic acid was increased in chromatogram B as compared with the corresponding peak in chromatogram A. This finding shows that part of the tetradecanoic acid was present

TABLE 2. Nature, relative retention times, fragments from mass spectrometry, and amounts of fatty acids present in lipopolysaccharides from *Fusobacterium nucleatum* Fev 1

Fatty acid	Relative t_R on gas-liquid chromatography ^a		Mass spectrometry		Amt in hydrolysates ^b (μ mol/mg)		
	Castor-wax (175°C)	EGSS-X (140°C)	Base peak	Characteristic peaks (m/e)	KOH	NaOCH ₃ (A)	NaOCH ₃ + diazomethane (B)
Tetradecanoic (C _{14:0})	1.00	1.00	74	242 ^b (M) ^c	0.258	0.130	0.226
Hexadecanoic (C _{16:0})	2.38	1.96	74	270 (M)	0.037	0.030	0.036
D-3-Hydroxytetradecanoic (3-OH-C _{14:0})	3.06	7.78	43	103, 240 (M-18), 208 (M-50)	0.204 ^d	0.141 ^e	0.195 ^e
D-3-Hydroxyhexadecanoic (3-OH-C _{16:0})	7.20	15	43	103, [268 (M-18)] ^f , 236 (M-50)	0.110		

^a Based on tetradecanoic acid methyl ester (t_R = 1.00).

^b Calculated for fatty acid methyl ester.

^c M, Molecular weight.

^d Sum of 3-OH-C_{14:0} and Δ^2 -C_{14:1} (0.072 μ mol/mg).

^e Sum of 3-OH-C_{14:0}, 3-OCH₃-C_{14:0} (0.079 μ mol/mg) and Δ^2 -C_{14:1} (0.009 μ mol/mg).

^f Not visible on mass spectrum.

in the free form, suggesting that it had been released from the lipopolysaccharide by β -elimination. It was concluded that 40% of the ester-bound 3-hydroxytetradecanoic acid in *F. nucleatum* Fev 1 lipopolysaccharide was 3-*O*-substituted by tetradecanoic acid. By analogy, it could be assumed that the smaller amounts of 3-hydroxytetradecanoic acid (0.05 μ mol/mg) found in the methanolysate originated from 3-*O*-(3-hydroxytetradecanoyl)-tetradecanoic acid. This, however, was unlikely since the amount of 3-methoxytetradecanoic acid methyl ester (0.079 μ mol/mg) detectable was lower than would be expected if the free 3-hydroxytetradecanoic acid had undergone β -elimination.

The (absolute) configuration of ester- and amide-bound 3-hydroxy fatty acids was determined by gas-liquid chromatography of diastereomeric derivatives (3-methoxy acid-L-phenylethylamides [20]). Gas-liquid chromatography of the derivatives on OV-1 (205°C) gave two main peaks with t_R values of 1.24 (peak 1) and 2.41 (peak 2) relative to tetradecanoic acid-L-phenylethylamide, which had a t_R value of 1.00. Authentic D- and L-3-methoxytetradecanoic acid-L-phenylethylamides revealed t_R values of 1.23 and 1.32, respectively; authentic D- and L-3-methoxyhexadecanoic acid-L-phenylethylamides had t_R values of 2.41 and 2.61, respectively. Accordingly, both 3-hydroxy acids present in *F. nucleatum* Fev 1 lipopolysaccharides possess the D-configuration.

Comparative fatty acid analyses were performed on other *Fusobacterium* strains (Table 3). The three *F. nucleatum* strains investigated (Fev 1, F1, and ATCC 10953) exhibited a similar fatty acid pattern in that D-3-hydroxyhexadecanoic acid was amide bound and tetradecanoic, hexadecanoic, and 3-hydroxytetradecanoic acids were ester bound, and in that part of the ester-bound 3-hydroxytetradecanoic acid was 3-*O*-acylated by tetradecanoic acid.

In lipopolysaccharides of *F. varium* ATCC 8501 and *F. mortiferum* VIP 0473, tetradecanoic, hexadecanoic, and D-3-hydroxytetradeca-

noic acids were detected, but 3-hydroxyhexadecanoic acid was not. This latter fatty acid therefore appears to be a characteristic constituent of *F. nucleatum* strains. In *F. mortiferum* and *F. varium*, part of the 3-hydroxytetradecanoic acid was amide bound. The other part as well as tetradecanoic and hexadecanoic acids was ester bound. Again, the ester-bound 3-hydroxytetradecanoic acid was partially 3-*O*-substituted with tetradecanoic acid.

DISCUSSION

According to the results of the present study, lipid A of *F. nucleatum* Fev 1 consists of β -1',6-linked D-glucosamine disaccharides, which carry two phosphate residues: one in glycosidic and one in ester linkage. This type of backbone structure has previously been identified in the majority of lipid A's from aerobic strains such as *Salmonella* (5, 6), *Escherichia coli* (6), *Shigella* (6, 17), and *Pseudomonas* (2). Lipid A of the anaerobic organism *Selenomonas ruminantium* also contains β -1',6-linked glucosamine disaccharides; phosphate, however, is absent (12).

The spectrum of fatty acids present in lipid A from *F. nucleatum* Fev 1 resembles that of enterobacterial and other lipid A's in that even-numbered, saturated, non-hydroxylated ($C_{14:0}$ and $C_{16:0}$) as well as 3-hydroxy acids (3-OH- $C_{14:0}$ and 3-OH- $C_{16:0}$) predominate. Of these, 3-hydroxyhexadecanoic acid is linked to the amino groups of the glucosamine residues. The other fatty acids are bound to the hydroxyl groups of the disaccharide backbone. Thus, like in most lipid A's studied, the 3-hydroxy acid with the longest chain is amide linked; the other hydroxy acids are present in ester bonds.

Part (approximately 40%) of the ester-bound 3-hydroxytetradecanoic acid was substituted by tetradecanoic acid. This structure, which is also present in enterobacterial lipid A's, was recognized by the fact that, with methylate, tetradecanoic acid is β -eliminated as the free acid and that concomitantly 3-methoxytetradecanoic

TABLE 3. Nature and relative amounts of fatty acids present in lipopolysaccharides of various *Fusobacterium* strains

Fatty acid	Relative amt (mol%) of fatty acids in:				
	<i>F. nucleatum</i> Fev 1	<i>F. nucleatum</i> F1	<i>F. nucleatum</i> ATCC 10953	<i>F. varium</i> ATCC 8501	<i>F. mortiferum</i> VIP 0473
$C_{14:0}$	39.1	23.9	36.4	33.0	27.5
$C_{16:0}$	6.2	7.6	5.0	7.7	18.9
D-3-OH- $C_{14:0}$	34.6 ^a	45.9	46.3	59.3 ^b	53.6
D-3-OH- $C_{16:0}$	<u>20.1</u>	<u>22.6</u>	<u>12.3</u>	Trace	

^a Values represent the sum of 3-OH- $C_{14:0}$ and Δ^2 - $C_{14:1}$.

^b Amide-bound fatty acids are underlined.

acid methyl ester is formed. In the methanolysate, small amounts of 3-hydroxytetradecanoic acid were also found, the origin of which is not well understood. That part of ester-bound 3-hydroxytetradecanoic acid was 3-*O*-substituted by 3-hydroxytetradecanoic acid appears to be unlikely since the amount of 3-methoxytetradecanoic acid methyl ester formed (0.079 $\mu\text{mol}/\text{mg}$) was too low to account for eliminated tetradecanoic acid (0.096 $\mu\text{mol}/\text{mg}$) plus 3-hydroxytetradecanoic acid (0.054 $\mu\text{mol}/\text{mg}$).

Configuration analysis of the 3-hydroxy fatty acids present in lipopolysaccharides of the various *Fusobacterium* strains investigated revealed that all 3-hydroxy acids possess, as was found in lipopolysaccharides of various other bacterial groups (20, 21), the *D*-configuration.

The fatty acid composition of other *F. nucleatum* strains was similar to that of *F. nucleatum* Fev 1 with regard to quantity, nature, and type of linkage of fatty acids. In *F. varium* and *F. mortiferum*, however, 3-hydroxyhexadecanoic acid was absent. In these cases only 3-hydroxytetradecanoic acid was present, which is, like in *Enterobacteria*, partly ester and partly amide bound. It should be mentioned that in lipopolysaccharides of two *F. necrophorum* strains, tetra- and hexadecanoic acids were detected; 3-hydroxy fatty acids, however, were absent (19).

The results of the present study indicate that the chemical structure of lipid A of *F. nucleatum* Fev 1 resembles that of lipid A of several other, systematically quite remote bacteria (e.g., *Salmonella* [6], *Pseudomonas* [2], *S. ruminantium* [12], and *Rhodopseudomonas gelatinosa* [6]) with regard to the glucosamine-phosphate backbone and the nature and type of linkage of the constituent fatty acids. It seems, therefore, that lipid A's among certain groups of gram-negative bacteria exhibit close structural relationships and that the lipid A component of these lipopolysaccharides was not subject to major modifications during evolution.

ACKNOWLEDGMENTS

The skillful technical assistance of Ulrike Pflugfelder and Inge Minner and the help of D. Borowiak and H. Mayer with mass spectrometry and of E. Warth and B. Kickhöfen with the amino acid analyzer are gratefully acknowledged. We are grateful to O. Lüderitz and O. Westphal for advice and encouragement.

This investigation was supported by grants from the Alexander von Humboldt-Stiftung (S.H.) and the Deutsche Forschungsgemeinschaft (E.T.R.).

LITERATURE CITED

1. de Araujo, W. C., E. Varah, and S. E. Mergenhagen. 1963. Immunochemical analysis of human oral strains of *Fusobacterium* and *Leptotrichia*. *J. Bacteriol.* 86:837-844.
2. Drewry, D. T., J. A. Lomax, G. W. Gray, and S. G.

- Wilkinson. 1973. Studies of lipid A fractions from the lipopolysaccharides of *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*. *Biochem. J.* 133:563-572.
3. Galanos, C., E. T. Rietschel, O. Lüderitz, and O. Westphal. 1971. Interaction of lipopolysaccharides and lipid A with complement. *Eur. J. Biochem.* 19:143-152.
4. Garcia, M. M., K. M. Charlton, and K. A. McKay. 1975. Characterization of endotoxins from *Fusobacterium necrophorum*. *Infect. Immun.* 11:371-379.
5. Gmeiner, J., O. Lüderitz, and O. Westphal. 1969. Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 6. Investigations on the structure of the lipid A component. *Eur. J. Biochem.* 7:370-379.
6. Hase, S., and E. T. Rietschel. 1976. Isolation and analysis of the lipid A backbone. Lipid A structure of lipopolysaccharides from various bacterial groups. *Eur. J. Biochem.* 63:101-107.
7. Hase, S., and E. T. Rietschel. 1976. Methylation analysis of glucosaminyl-glucosaminyl-glucosaminyl disaccharides. Formation of 2-deoxy-2-(*N*-acetylacetamido)-glucitol-derivatives. *Eur. J. Biochem.* 63:93-99.
8. Hofstad, T. 1974. The distribution of heptose and 2-keto-3-deoxyoctonate in *Bacteroidaceae*. *J. Gen. Microbiol.* 85:314-320.
9. Hofstad, T., and T. Kristoffersen. 1970. Chemical characteristics of endotoxin from *Bacteroides fragilis* NCTC 9343. *J. Gen. Microbiol.* 61:15-19.
10. Hofstad, T., T. Kristoffersen, and K. A. Selvig. 1972. Electron microscopy of endotoxin lipopolysaccharides from *Bacteroides*, *Fusobacterium* and *Sphaerophorus*. *Acta Pathol. Microbiol. Scand. Sect. B* 80:413-419.
11. Ikawa, M., J. B. Koepfli, S. C. Mudd, and C. Niemann. 1953. An agent from *E. coli* causing hemorrhage and regression of experimental mouse tumor. III. The component fatty acids in the phospholipid moiety. *J. Am. Chem. Soc.* 75:1035-1038.
12. Kamio, Y., K. C. Kim, and H. Takahashi. 1971. Chemical structure of lipid A of *Selenomonas ruminantium*. *J. Biochem. (Tokyo)* 70:187-191.
13. Kristoffersen, T. 1969. Immunochemical studies of oral *Fusobacteria*. I. Major precipitinogens. *Acta Pathol. Microbiol. Scand.* 77:235-246.
14. Kristoffersen, T., and T. Hofstad. 1970. Chemical composition of lipopolysaccharide endotoxins from human oral *Fusobacteria*. *Arch. Oral Biol.* 15:909-916.
15. Kristoffersen, T., J. A. Maeland, and T. Hofstad. 1971. Serologic properties of lipopolysaccharide endotoxins from oral *Fusobacteria*. *Scand. J. Dent. Res.* 79:105-112.
16. Lüderitz, O., O. Westphal, A. M. Staub, and H. Nikaido. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides, p. 145-233. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), *Microbial toxins*, vol. 4. Academic Press Inc., New York.
17. Lugowski, C., and E. Romanowska. 1974. Chemical studies on *Shigella sonnei* lipid A. *Eur. J. Biochem.* 48:319-323.
18. Mega, T., T. Ikenaka, and Y. Matsushima. 1970. Studies on *N*-acetyl- β -D-glucosaminidase of *Aspergillus oryzae*. I. Purification and characterization of *N*-acetyl- β -D-glucosaminidase obtained from takadiastase. *J. Biochem. (Tokyo)* 68:109-117.
19. Meisel-Mikolajczyk, F., and T. Dobrowolska. 1974. Comparative immunochemical studies on the endotoxins of two *Fusobacterium necrophorum* strains. *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* 22:555-562.
20. Rietschel, E. T. 1976. Absolute configuration of 3-hydroxy fatty acids present in lipopolysaccharides from various bacterial groups. *Eur. J. Biochem.* 64:423-428.

21. Rietschel, E. T., C. Galanos, and O. Lüderitz. 1975. Structure, endotoxicity, and immunogenicity of the lipid A component of bacterial lipopolysaccharides, p. 307-314. In D. Schlessinger (ed.), *Microbiology-1975*. American Society for Microbiology, Washington, D.C.
22. Rietschel, E. T., H. Gottert, O. Lüderitz, and O. Westphal. 1972. Nature and linkage of the fatty acids present in the lipid A component of *Salmonella* lipopolysaccharides. *Eur. J. Biochem.* 28:166-173.
23. Rooney, S. A., H. Goldfine, and C. C. Sweeley. 1972. The identification of trans-2-tetradecenoic acid in hydrolysates of lipid A from *Escherichia coli*. *Biochim. Biophys. Acta* 270:289-295.
24. Stellner, K., H. Saito, and S. Hakomori. 1973. Determination of aminosugar linkages in glycolipids by methylation. Aminosugar linkages of ceramide pentasaccharides of rabbit erythrocytes and of Forssman-antigen. *Arch. Biochem. Biophys.* 155:464-472.
25. Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraktion von Bakterien mit Phenol-Wasser. *Z. Naturforsch.* 7b:148-155.